Expert Opinion

Therapeutic peptides for cancer therapy. Part II – cell cycle inhibitory peptides and apoptosis-inducing peptides

Drazen Raucher†, Shama Moktan, Iqbal Massodi & Gene L Bidwell III
The University of Mississippi Medical Center, Department of Biochemistry, 2500 North State Street, Jackson, MS 39216, USA

Background: Therapeutic peptides have great potential as anticancer agents owing to their ease of rational design and target specificity. However, their utility *in vivo* is limited by low stability and poor tumor penetration. *Objective*: The authors review the development of peptide inhibitors with potential for cancer therapy. Peptides that arrest the cell cycle by mimicking CDK inhibitors or induce apoptosis directly are discussed. *Methods*: The authors searched Medline for articles concerning the development of therapeutic peptides and their delivery. *Results/conclusion*: Inhibition of cancer cell proliferation directly using peptides that arrest the cell cycle or induce apoptosis is a promising strategy. Peptides can be designed that interact very specifically with cyclins and/or cyclin-dependent kinases and with members of apoptotic cascades. Use of these peptides is not limited by their design, as a rational approach to peptide design is much less challenging than the design of small molecule inhibitors of specific protein–protein interactions. However, the limitations of peptide therapy lie in the poor pharmacokinetic properties of these large, often charged molecules. Therefore, overcoming the drug delivery hurdles could open the door for effective peptide therapy, thus making an entirely new class of molecules useful as anticancer drugs.

Keywords: Bcl-2, drug delivery, p16, p21, p27, Smac, therapeutic peptide

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1. Introduction

Therapeutic peptides (TP) have captured interest as a potential new class of drugs. TPs are attractive from a drug design standpoint because they can be easily designed and produced, and they can be very specific for their targets. Design of TPs is aided by our growing knowledge of protein sequences, structures and interacting partners. Therefore, peptides can be generated that contain the important amino acids from a protein–protein surface contact, and these peptides can serve to inhibit or even mimic the protein–protein interactions from which they were modeled. However, the use of TPs as drugs is hampered by the challenges of delivering these large, often charged, and labile molecules *in vivo* [1,2]. Particularly for cancer therapy, accumulation of effective doses of peptides at a tumor site is limited by the rapid degradation and poor tumor cell penetration of these molecules. Therefore, advancement of TPs for therapy of cancer as well as other disorders requires innovation of new delivery technologies. As discussed below, these limitations can be addressed by introducing non-natural amino acids into the peptides to increase stability and by attaching the peptides to macromolecular carriers to improve both stability and delivery.

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As these peptides have intracellular targets, they need to gain entry across the plasma membrane in order to function. The most common strategy applied for intracellular delivery is to attach the cargo peptide to a cell penetrating peptide (CPP). CPPs are short peptides, usually rich in either basic amino acids or hydrophobic amino acids, which are capable of delivering cargo across plasma membranes. The most commonly used CPPs are the penetratin peptide from the *Drosophila* antennapedia protein [3], the Tat peptide from the HIV-1 Tat protein [4] and the membrane translocating sequence (MTS) from Kaposi's fibroblast growth factor [5]. The relative efficiencies and cell entry mechanism of these peptides have been reviewed previously [6-8].

In general, TPs can be grouped into three classes: peptides that inhibit signal transduction pathways; peptides that arrest the cell cycle; and peptides that directly induce apoptosis or necrosis. Signal transduction inhibitors were discussed in Part I of this review series, and this article focuses on cell cycle-inhibiting and apoptosis-inducing peptides. The cell cycle inhibitory peptides function by modulating cyclin and cyclin-dependent kinase (Cdk) activity, either by mimicking the function of the natural CDK inhibitor proteins p21, p16 and p27, or by modulating the cyclin/Cdk complexes or their targets directly. The apoptosis-inducing peptides function by inhibiting anti-apoptotic members of the Bcl-2 family of proteins, by mimicking the pro-apoptotic Bcl-2 family members, or by modulating caspase activity. Also, there is a very heterogeneous class of peptides, mostly derived from natural toxins, which lyse the plasma membranes of cells and thereby induce necrosis. These lytic peptides have been reviewed previously [9] and are not discussed here.

2. Body

2.1 Peptide inhibitors of cell cycle progression

Progression through the cell cycle is controlled by Cdks, which, when bound to cyclins, phosphorylate target proteins that control cell cycle-specific events such as chromosome condensation, nuclear membrane breakdown, spindle formation, and so on. Cdks are activated by cyclin binding and by phosphorylation by other Cdk-activating kinases (CAKs), and they are inhibited by phosphorylation of inhibitory sites by the Wee1 kinase and by the binding of Cdk inhibitor proteins (CKIs). In mammals, there are two main classes of CKI: the KIP/CIP family of CKIs, which includes p21 and p27, and the INK4 proteins, which include p16. p21 inhibits Cdk2/cyclin E and Cdk2/cyclin A, p16 inhibits Cdk4/6/cylin D, and p27 inhibits Cdk2/cyclin E and Cdk2/cyclin A (**Figure 1**). p21 is a p53-controlled gene that is activated during the DNA damage response pathway, and loss of p21 activity (due to either p21 mutations or loss of p53 function) causes cells to lose the ability to arrest the cell cycle following DNA damage. p16 is encoded by the *INK4a* gene, which is very often inactivated in a large variety of tumor cell lines. In fact, *INK4a* inactivation is the second most common genetic event (following p53 mutation or deletion) in tumorigenesis [10]. Therefore, restoration of CKI activity in cells that have lost it may be a promising method for cancer therapy.

2.1.1 *p21 peptides*

As mentioned above, p21 can bind and inhibit the cyclin/Cdk, which in turn prevents phosphorylation of the Rb protein. When unphosphorylated, Rb binds and inhibits the proliferation-promoting transcription factor E2F. In addition, p21 can also bind to proliferating cell nuclear antigen (PCNA), which is a processivity factor for DNA polymerase δ (**Figure 1**). Two classes of p21-derived peptides have been described: N-terminal peptides, which inhibit cyclin/Cdk activation, and C-terminal peptides, which interact with PCNA and inhibit DNA replication [11].

Chen *et al.* synthesized overlapping peptides spanning the entire p21 sequence, and they found that a peptide containing amino acids 15 – 40 and, to a lesser extent, a peptide containing amino acids 58 – 77, were capable of blocking the p21-dependent inhibition of Cdk2/cylin E [12]. This indicates that these regions of p21 are important for binding to Cdk2/cyclin E. Bonfanti *et al.* applied this knowledge by making fusions of peptides from the regions identified by Chen with penetratin [13]. They determined that when fused to the CPP, peptides spanning amino acids 17 – 33 (**Table 1**, peptide 1) or 63 – 77 (**Table 1**, peptide 2) inhibited the proliferation of the human ovarian cancer cell line SKOV-3 (which carries a p53 mutation) with IC_{50}s of 38 and 90 μM, respectively, and similarly inhibited proliferation of the IGROV-1 (p53 wild type) ovarian cancer cell line with IC_{50}s of 27 and 54 μM, respectively. The increased potency of the 17 – 33 peptide over the 63 – 77 peptide is consistent with Chen's findings that the 15 – 40 peptide was more important for p21 binding to the Cdk/cyclin than the 58 – 77 peptide. The authors went on to confirm that the peptides were functioning by inhibiting Cdk1 and Cdk2 activity in the cultured cells.

Using a similar peptide scanning technique, Warbrick *et al.* demonstrated that p21 amino acids 141 – 160 (**Table 1**, peptide 3) were capable of binding directly and specifically to PCNA [14], a result confirmed by Chen *et al.* for amino acids 139 – 164 [12] and by Pan *et al.* for amino acids 139 – 160 [15]. Pan further demonstrated that the 139 – 160 peptide was capable of inhibiting the repair of UV-damaged DNA in HeLa cell extracts, which is consistent with its ability to bind and sequester PCNA. Warbrick narrowed the critical region for PCNA binding to amino acids 141 – 152, and, by alanine scanning, showed that mutation of M147 or F150 completely abolished PCNA binding. Independently, Ball *et al.* reported that the 141 – 160 peptide binds to and inhibits Cdk4/cyclin D1 (note that this is independent of the N-terminal Cdk2/cyclin E binding motif) [16]. Using similar techniques, they determined that the minimum Cdk4/cyclin D1 binding motif was contained between

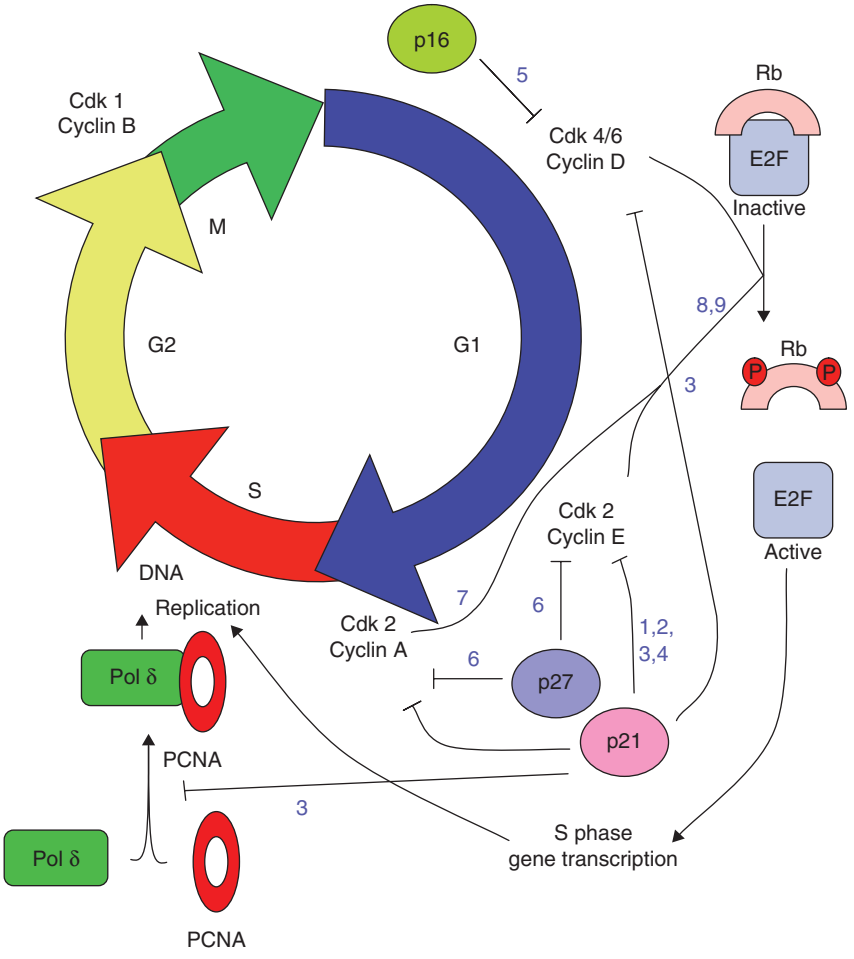

Figure 1. Cell cycle inhibitory peptides. Progression through the cell cycle is controlled by the interactions of cyclins and Cdks. The cell cycle can be blocked by Cdk inhibitors, and the site of action of each peptide-based Cdk inhibitor or other inhibitory peptide is indicated by the numbers, which refer to the peptide number in **Table 1** and in the text.

amino acids 155 – 160, and that L157 and F159 were absolutely critical. In summary, the C-terminal region between amino acids 141 and 160 contains binding motifs for both PCNA (141 – 152) and Cdk4/cyclin D1 (155 – 160). Therefore, use of the full-length 141 – 160 peptide as an inhibitor may lead to two separate mechanisms of action. When Ball *et al.* fused a mutant form of the 141 – 160 (M147A) peptide to penetratin [16], they demonstrated that it inhibited proliferation of human keratinocyte-derived HaCaT cells (mutant p53). The M147A mutation abolishes PCNA binding [14], which allowed the authors to study only the Cdk4/cyclin D1 inhibitory effects of the peptide. This peptide induced cell cycle arrest in G1 phase and prevented Rb phosphorylation. The native 141 – 160 peptide fused to penetratin arrested the cell cycle in DLD1 colon cancer cells in a mechanism that was dependent on PCNA binding [17]. Mutoh *et al.* showed that in addition to the previously reported inhibition of Cdk4/cyclin D1, a penetratin-fused peptide spanning amino acids 139 – 164 (**Table 1**, peptide 4) could also inhibit Cdk2/cylin E, and this peptide induced necrosis in CA46 lymphoma cells [18].

In an attempt to enhance the stability of the peptides in the intracellular environment, Mattock expressed the 141 – 160 peptide as a fusion with GFP in several human cancer cell lines [19]. They demonstrated that the GFP–peptide fusion is capable of interacting with Cdk2/cyclin E and inhibiting its activity. They also used a GFP-fused mutant peptide (Q144A, M147A, F150A) that is unable to bind PCNA, and this peptide was equally efficient at inhibiting the Cdk2/cylin E activity. The GFP-fused wild-type peptide inhibited proliferation of H1299 non-small-cell lung carcinoma cells (p53 deletion), U2OS osteosarcoma cells (p53 wild type), and Saos2 osteocarcinoma cells (p53 deletion) as judged by a clonogenic assay. The GFP-fused mutant peptide had no effect in these cell lines, indicating that inhibition was dependent on the peptide's ability to bind PCNA. However, both the wild type and mutant GFP-fused peptide inhibited proliferation of HeLa cells, and the authors speculated that the peptides may

Table 1. Cell cycle inhibitory and pro-apoptotic peptides.

Class	Peptide number	Peptide name	Protein of origin	Amino acids	Sequence	Validation	Ref.
Cell cycle inhibitors							
	1	n.s.*	p21	17 – 33	ACRRLFGPVDSEQLSRD	*In vitro*	[13]
	2	n.s.	p21	63 – 77	AWERVRGLGLPKLY	*In vitro*	[13]
	3	Peptide 10	p21	141 – 160	KRRQTSMTDFYHSKRRLIFS	*In vitro*	[14]
	4	W10	p21	139 – 164	GRKRRQTSMTDFYHSKRRLIFSKRKP	*In vitro*	[18]
	5	Peptide 6	p16	84 – 103	DAAREGFLDTLVVLHRAGAR	*In vivo*	[28]
	6	Peptide 5a	p27	Modified from 30 – 34	AA-Abu‡-RKLFG §	*In vitro*	[38]
	7	C4	Cyclin A	285 – 306	TYTKKQVLRMEHLVLKVLTFDL	*In vitro*	[39]
	8	n.s.	E2F	87 – 64	PVKRRLDL	*In vitro*	[40]
	9	n.s.	Rb	864 – 880	SNPPKPLKKLRFDIE	*In vitro*	[42]
Apoptosis inducers							
	10	Akt-*in*	TCL1	10 – 24	AVTDHPDRLWAWEKF	*In vivo*	[52]
	11	Peptide2	FKHRL1	16 – 24	VELDPEFEPRARERTYAFGH	*In vitro*	[53]
	12	n.s.	Bak	72 – 87	GQVGRQLAIIGDDINR	*In vivo*	[56]
	13	TO4	Bax	52 – 72	QDASTKKLSECLKRIGDELDS	*In vitro*	[55]
	14	n.s.	Bax	53 – 86	DASTKKLSECLKRIGDELDSNMELQRMIAAVDTD	*In vitro*	[71]
	15	Cpm-1285	Bad (*Mus musculis*)	140 – 165	NLWAAQRYGRELRRMSDEFEGSFKGL	*In vivo*	[74]
	16	n.s.	Bad	103 – 127	NLWAAQRYGRELRRMSDEFVDSFKK	*In vitro*	[75]
	17	BH3 BAD	Bad	103 – 123	NLWAAQRYGRELRRMSDEFVD	*In vivo*	[77]
	18	Bim	Bim	145 – 165	EIWIAQELRRIGDEFNAYYAR	*In vivo*	[79]
	19	n.s.	Bid	84 – 99	RNIARHLAQVGDSMDR	*In vitro*	[57]
	20	^{SAHB}A	Bid	80 – 101	EDIIRNIARHLAQ¶VGD¶SN#DRSIW	*In vivo*	[80]
	21	Smac-7	Mature Smac	1 – 7	AVPIAQK	*In vivo*	[83]
	22	n.s.	Mature Smac	1 – 4	AVPI	*In vitro*	[84]
	23	dAVPI	Mature Smac	1 – 4	Dimeric AVPI	*In vitro*	[95]

*n.s., name not specified.
‡Abu, 2-aminobutyric acid.
§Site of cyclization.
¶Site of hydrocarbon staple.
#L-amino acid.

be interacting with the HPV-16 E7 protein, which is expressed in HeLa cells, and interrupting contacts important for its function.

The authors' lab has also designed a carrier for a p21 peptide (the 139 – 164 peptide used by Mutoh, **Table 1**, peptide 4) in which the peptide is fused to the thermally responsive Elastin-like polypeptide (ELP) carrier. ELP has the unique property of being soluble in aqueous solution below a characteristic transition temperature (T_t), but forming aggregates when the temperature is raised above the T_t [20]. Fusion of peptides to an ELP carrier has several advantages. First, ELP is a macromolecule with a long plasma half-life [21], and fusing it to a TP might serve to stabilize the TP in circulation. Second, the heat-induced aggregation of ELP can be exploited for drug delivery purposes. When injected systemically, ELP can be concentrated at the site of a tumor by selectively heating the area [22-24]. Third, ELP can be expressed in *Escherichia coli* and, owing to its heat-induced aggregation, is easily purified by centrifugation above the T_t. This makes generation of large amounts of purified polypeptide very easy. For delivery of the p21 peptide, the coding sequence of ELP was modified at the N terminus with the penetratin CPP to facilitate cellular uptake of this macromolecule and at the C terminus with the p21 139 – 164 peptide (Pen-ELP-p21). The authors demonstrated that the Pen-ELP-p21 polypeptide, but not a control polypeptide lacking the p21 sequence, inhibited proliferation of HeLa and SKOV-3 cells [25]. More recently, a more potent version of the ELP-delivered p21 peptide was developed in which the Bac CPP [26] is used. This polypeptide is capable of localizing to the nucleus of SKOV-3 and Panc-1 pancreatic cancer cells, arresting the cell cycle, preventing Rb phosphorylation, and inducing apoptosis [27]. Furthermore, the antiproliferative effect of this polypeptide can be increased by increasing its intracellular delivery using hyperthermia treatment.

2.1.2 p16 peptide

In an effort to restore p16 function to cancer cells that have mutated or deleted p16 genes, Fåhraeus *et al.* developed a 20-amino acid peptide from p16 residues 84 – 103 (**Table 1**, peptide 5) that is capable of interacting with Cdk4 and Cdk6 [28]. This peptide blocked Rb phosphorylation by Cdk4/cyclin D1 *in vitro* (**Figure 1**), and a penetratin fusion of the peptide blocked cell cycle progression in HaCaT cells. The authors determined by cell synchronization that the peptide was functioning during late G1 phase to restrict S phase entry. They further determined that this peptide blocked S phase entry in MCF-7 breast cancer cells, MRC-5 human fibroblasts, 3T3 mouse fibroblasts and HT-29 human colon carcinoma cells, but was unable to arrest the cell cycle in the Rb negative cell line Saos-2 [29]. Fujimoto *et al.* demonstrated the ability of the penetratin-fused p16 peptide to arrest the cell cycle and inhibit proliferation in p16 negative, Rb positive pancreatic cancer cells [30]. The same group also tested the penetratin-p16 peptide *in vivo* in a mouse model of pancreatic cancer in which two pancreatic cell lines were grown both subcutaneously (s.c.) and intraperitoneally (i.p.). The penetratin-p16 peptide was administered systemically by daily i.p. injections for 3 weeks. This treatment, but not treatment with the control penetratin or p16-only peptides, significantly reduced the tumor size in two s.c. prostate tumor models. When examined histologically, the tumors showed hallmarks of apoptosis, including vacuole formation inside the tumor and positive TUNEL staining. The same treatment conditions also induced a survival benefit of 6 days mean survival greater than control in a pancreatic tumor peritoneal dissemination model. Finally, no effects of peptide treatment were seen on the animal's body weight, blood counts, or major organ histology [31].

Kondo *et al.* described an alternative method for intracellular delivery of a shortened version of the p16 peptide to leukemia and lymphoma cells. The authors first fused r9 (a CPP consisting of a string of nine D-Arg residues) to the p16 peptide. Next, they generated a new peptide transporter, which they termed Wr-T. Wr-T was based on the Pep-1 CPP, but an extra hydrophobic pocket was added to mediate binding to the p16 peptide, and the SV40 nuclear localization signal present in the original Pep-1 was replaced with nine D-Arg residues. For cell treatment, the Wr-T and r9-p16 peptides were simply mixed, and the authors demonstrate that the combination was far more efficient than r9-p16 alone for cellular uptake. Furthermore, the combination of peptides inhibited the proliferation of several leukemia and lymphoma cell lines that lack p16 expression, including primary lymphoma cells isolated from a patient's pleural effusion. Finally, the peptide mixture, when injected directly at the tumor site, was capable of slowing the proliferation of Burkitt's lymphoma cells grown s.c. in nude mice [32]. The group further applied the Wr-T delivery strategy for treatment of glioblastoma cell lines with a mixture of the r9-p16 peptide and a modified version of the p14 peptide discussed in Part I (Section 2.4, [33]). The combination of the two peptides was much more effective than individual peptide treatment for inhibition of proliferation and induction of apoptosis in a human glioblastoma cell line. Furthermore, injection of the Wr-T-delivered p14 and p16 peptide mixture into the right ventricle of the heart in nude mice bearing intracerebral tumors led to peptide accumulation at the tumor site, and mice treated with the combination peptide therapy lived 1.5 times longer than saline-treated controls before succumbing to hemispheric occupying tumors [34].

In an interesting application, Fåhraeus and Lane also reported that treatment of cells that express $\alpha_v\beta_3$–integrin with the p16 peptide or the p21 141 – 160 peptide described above blocked their ability to spread and migrate on a vitronectin-coated substrate [35]. The inhibition was specific to the $\alpha_v\beta_3$–vitronectin interaction. The authors concluded that inhibition was due to the peptide's ability to inhibit a cytoplasmic fraction of Cdk6 that was later identified to

play a role in cell-spreading by modulating cytoskeletal dynamics [36]. This effect was also linked to the p16 peptide's ability to prevent tube formation in cultures of HUVECs, indicating a possible application for these peptides in anti-angiogenesis therapy [37]. These results indicate that the CKI-derived peptides may be useful not only for direct cell cycle arrest, but also for treatment of metastatic cancers where cell spreading, migration and angiogenesis are important concerns.

2.1.3 *p27 peptide*

In an attempt to inhibit the Cdk 2/cyclin A and Cdk 2/cyclin E complexes, a peptide was generated based on the cyclin recognition motif of p27. Using a clever approach to increase peptide stability, Andrews *et al*. generated cyclic peptides based on the p27 sequence by joining the terminal Gly residue to the side chain of an internal residue (**Table 1**, peptide 6) [38]. Although the linear version of the peptide was relatively inactive, the cyclic peptide based on the cyclin recognition motif of p27 was a very potent inhibitor of both cyclin A and Cdk 2 binding and of Rb phosphorylation by Cdk 2/cyclin A (**Figure 1**).

2.1.4 *Peptide from cyclin A*

Using the crystal structure of the Cdk2/cyclin A complex, Gondeau *et al*. synthesized a peptide containing amino acids 285 – 306 of cyclin A, which were known to form contacts with Cdk2 [39]. This peptide prevented Cdk2/cyclin A-mediated phosphorylation of Rb and Histone H1. Furthermore, while performing an alanine scan to determine the critical residues for kinase inhibition, the authors found that replacement of Glu295 with Ala produced a peptide (called C4) that was more soluble and a twofold more potent inhibitor of Cdk2/cyclin A activity (**Table 1**, peptide 7). The authors determined that the C4 peptide binds to the Cdk2/cyclin A complex and inhibits its activity. Finally, when delivered into MDA-MB-231, Jurkat, MCF-7 and HepG2 cells by fusion with the Tat peptide, the C4 peptide efficiently inhibited proliferation in all cell lines, with IC_{50}s ranging from 2 to 14.2 μM.

2.1.5 *Peptides from Rb and E2F*

In an attempt to identify the critical residues for Cdk2/cylin A binding to E2F, Adams *et al*. screened short peptides from the cyclin/Cdk binding domain of E2F. The authors identified an 8-residue peptide (**Table 1**, peptide 8) spanning amino acids 87 – 94 of E2F that was capable of preventing Cdk2/cyclin A–E2F binding [40]. This peptide prevented Rb phosphorylation by Cdk2/cyclin A *in vitro*. Furthermore, the authors identified related sequences in other cyclin/Cdk2-binding proteins, including p21 and p27, and proposed this domain as a general cyclin/Cdk binding motif. The putative cylcin/Cdk binding motifs described by Adams are in agreement with the other reports described here. The two regions from p21 described as being important for cyclin/Cdk binding by Adams are contained in the N-terminal peptide described by Bonfanti and the C-terminal peptide described by Warbrick, and the cyclin/Cdk-binding domain predicted by Adams in p27 is included in the cyclic peptide later used by Andrews. In a follow-up study, the E2F peptide was fused to the Tat and penetratin CPPs, and the cell-permeable versions of the peptide inhibited the proliferation of U2OS cells and induced signs of apoptosis [41]. Importantly, the CPP-fused E2F peptide did not affect the proliferation of the non-transformed HaCaT keratinocytes, Rat1A fibroblasts, or WI-38 fibroblasts, and the authors concluded that immortalized and transformed cells differ qualitatively in their response to cyclin/Cdk2 inhibition. Adams *et al*. also identified five sequences homologous to the cylcin/Cdk binding region of E2F in the C terminus of the Rb protein. After identifying the exact region important for cyclin/Cdk binding, a peptide was generated that spanned amino acids 864 – 880 of Rb (**Table 1**, peptide 9). This peptide prevented Rb phosphorylation by Cdk2/cyclin A [42], although not as potently as the E2F-derived peptide.

2.2 Peptides that induce apoptosis

Apoptosis plays a crucial role in the elimination of cells in which DNA is damaged and cannot be repaired. Cancer cells frequently contain inactivated apoptotic pathways, which result in uncontrolled cell growth. Therefore, there is a great interest in developing small molecules and therapeutic peptides for selectively inducing apoptosis in cancer cells. This review focuses on pro-apoptotic peptides that target protein kinase B (Akt kinase), the anti-apoptotic Bcl-2 family proteins, and the cellular inhibitors of apoptosis (IAPs).

Protein kinase B, also known as Akt, is a serine/threonine kinase [43,44]. Complete phosphorylation and activation of Akt requires membrane anchoring by means of its pleckstrin homology (PH) domain [45]. Activated Akt phosphorylates and inhibits key apoptosis proteins (**Figure 2**) [46-48]. Akt also regulates NF-κB-mediated transcription of IAPs [49]. These events lead to the protection of cells against apoptosis and enhancement of malignant cell growth. Disrupting Akt interaction with the cell membrane or directly inhibiting its ability to phosphorylate it substrates is a viable approach for inhibiting Akt-mediated protection against apoptosis. Although Akt is a signaling molecule, Akt peptides will be discussed along with the apoptosis-inducing peptides because of their role in the apoptotic program.

The Bcl-2 family consists of the anti-apoptotic and the pro-apoptotic proteins (**Figure 2**). They play a central role in the regulation of apoptosis. The fluctuation in the level of these two opposing members essentially determines the fate of the cell. Normal cells maintain a healthy balance between these two protein groups. However, anti-apoptotic proteins are overexpressed in cancer cells, allowing the survival and proliferation of cancer cells. Therefore, targeting the anti-apoptotic members of the Bcl-2 family in cancer cells is an effective therapeutic for the treatment of cancer. All

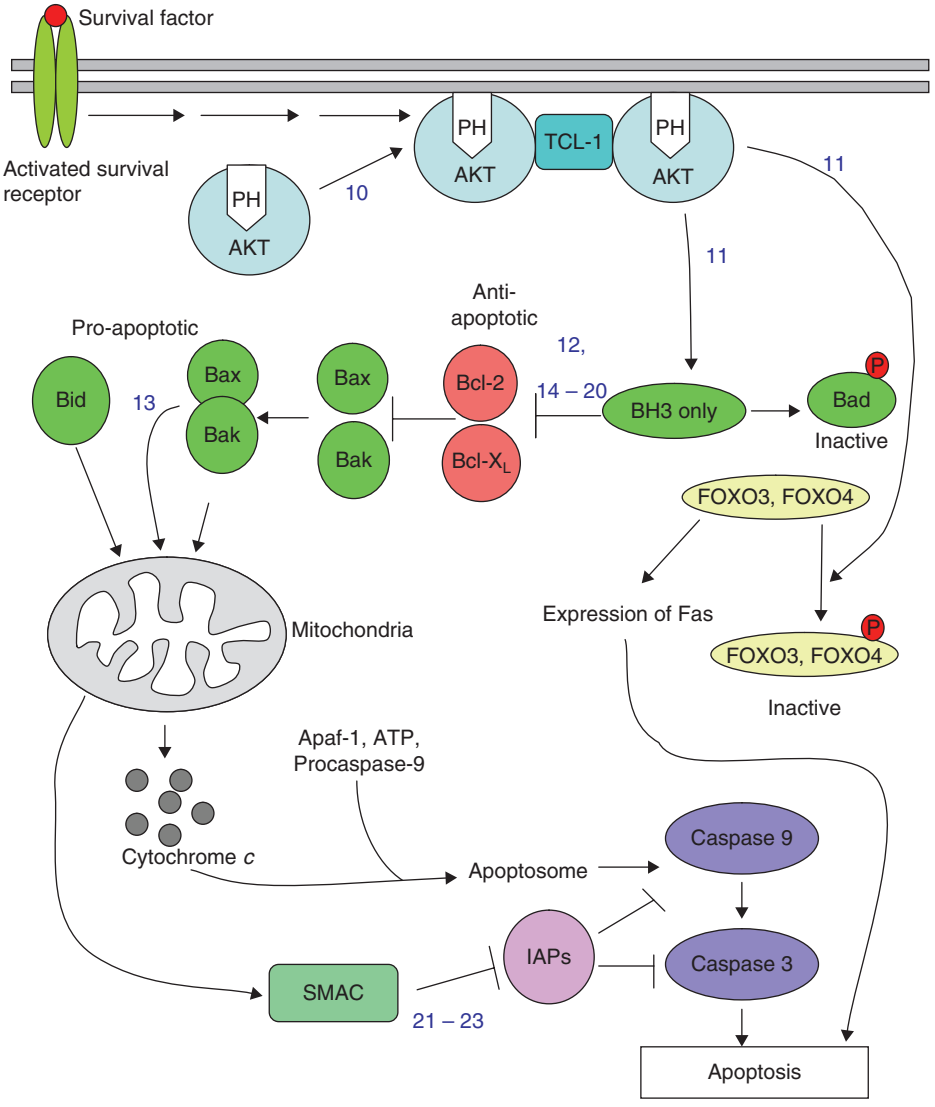

Figure 2. Apoptosis-inducing peptides. Apoptosis can be induced by cytochrome *c* release from mitochondria, which is driven by members of the Bcl-2 family or proteins, or directly through activation of Fas expression. These processes can be stimulated by peptides, and the site of action of each peptide is indicated by the numbers, which refer to the peptide numbers in **Table 1** and in the text.

Bcl-2 family proteins share the Bcl-2 homology 3 (BH3) domain. The pro-apoptotic Bcl-2 proteins are grouped into either the BH3-only proteins (Bad, Bik, Bim and Bid) or the BH1 – BH3 multi-domain proteins (Bax, Bak). The BH3 domain is crucial for homodimer and/or heterodimer formation with the anti-apoptotic Bcl-2 family proteins, which determines either repression or induction of apoptosis (reviewed in [50]). Short peptides derived from the BH3 domain of either the BH3-only or the BH1 – BH3 multi-domain proapoptotic proteins have been shown to induce apoptosis in various cancer cells. They are discussed in this review.

Cellular inhibitors of apoptosis block apoptosis at the initiator caspase 9 as well as at the effector caspases 3 and 7 (**Figure 2**). IAPs are regulated by the endogenous inhibitor called the second mitochondrial activator of caspases (Smac, also known as DIABLO). IAPs are overexpressed in some cancer cells, which make these proteins an attractive target for therapy. Smac/DIABLO offers a basis for the design of therapeutic peptides that bind to IAPs and inhibit caspase binding.

2.2.1 *Akt inhibitory peptides*

The PH domain of Akt is crucial for its recruitment to the cell membrane, where it is activated by phosphoinositol-dependent kinase 1 (PDK). Furthermore, the proto-oncogene TCL1 1 (T-cell leukemia 1) has been implicated as a co-activator of Akt [51]. Interaction of TCL1 with the PH domain of Akt and subsequent dimerization of TCL1 facilitates transphosphorylation of Akt dimers. A short peptide (**Table 1**,

peptide 10) derived from the Akt-binding βA strand of TCL1 binds to the PH domain of Akt and is effective in inhibiting Akt kinase activity [52]. Effective cellular delivery of this Akt-*in* peptide was achieved by fusing the peptide with the cell-penetrating Tat peptide. By interacting with the full-length Akt, particularly the PH domain of Akt, Tat-Akt-*in* specifically inhibited Akt activation in a dose-dependent manner. Lipid-protein pull-down assays confirm that Akt-*in* blocked the PH domain of Akt from translocating to the cell membrane and being activated by PDK1. Most importantly, the phosphorylation of critical Ser/Thr residues, which is required for the activation of Akt, did not occur in the presence of Akt-*in*. This inhibition of Akt activation was maintained even after stimulation of cells by platelet-derived growth factors. Subsequently, Akt-*in* prevented Akt-mediated phosphorylation of downstream substrates such as GSKα and the pro-apoptotic protein, Bad. Therefore, Akt-*in* promoted cell death as seen in the reduction of cell viability of the human T-cell line T4. Moreover, this inhibitory peptide also had an antitumor effect in QRsP-11 fibrosarcoma tumor bearing C57BL/6 mice, as evidenced by the reduction of tumor volume and the presence of apoptotic cells and unphosphorylated Akt in the tumors in the Akt-*in* treatment group. It is important to note here that the antiproliferative and the antitumor effect of Akt-*in* are attained only when it is coupled to the Tat peptide. This underscores the importance of intracellular delivery of TPs.

Luo *et al.* designed a chimeric peptide (**Table 1**, peptide 11) based on the Akt binding region of the forkhead transcription factor (FKHRL1) and the minimal binding motif of other Akt substrates [53]. FKHRL1 is one of the endogenous substrates of Akt. The model pseudosubstrate peptide contained a putative serine phosphorylation residue. Mutation of serine to alanine increased the inhibitory activity of the pseudosubstrate peptide as seen by the decreased kinase activity of Akt. As evidence that this inhibitory kinase activity is not global, the pseudosubstrate peptide inhibited only Akt kinase activity, not the activity of other kinases of the AGC family to which Akt belongs. Sustained inhibition of phosphorylation of the Akt target GSK3 in cells required continuous delivery of the peptide into the cells by fusion with poly-D-arginine. This translated to inhibition of cell growth.

2.2.2 BH3 peptides from the multi-domain pro-apoptotic Bcl-2 proteins

The BH1 – BH3 multi-domain pro-apoptotic proteins include Bak and Bax. Bak oligomerizes with Bax to mediate the release of cytochrome *c* from the mitochondria to induce apoptosis (**Figure 2**). The anti-apoptotic proteins Bcl-2 and Bcl-X_L heterodimerize with Bax and Bak, which curbs their apoptotic activity, resulting in the suppression of apoptosis [54]. The BH3 region is crucial for binding of Bcl-2 family proteins. The binding of a combination of pro-apoptotic and anti-apoptotic Bcl-2 proteins essentially modulates apoptosis. Releasing Bak and Bax from the inhibitory grasps of Bcl-2 and Bcl-X_L has been the basis of design of BH3 peptides that promote apoptosis.

2.2.2.1 BH3 peptide from Bak

One of the early quantitative binding studies done on member proteins of the Bcl-2 family identified 16 – 21-mer peptides from the BH3 region of Bax and Bak to be effective in inhibiting Bcl-2 and Bcl-x_L homodimerization as well as Bcl-2 or Bcl-x_L heterodimerization with Bax [55]. Structural studies showed that a 16-mer peptide from the BH3 region of Bak (**Table 1**, peptide 12) binds to Bcl-2 by adopting an α-helix conformation through specific hydrophobic and electrostatic interactions [56]. In *Xenopus* egg extract, this peptide caused a rapid increase in caspase activation [57]. As evidence that this is not due to nonspecific interactions, a mutant Bak-BH3 peptide did not induce caspase activation. Further information on the pro-apoptotic property of Bak-BH3 was gained from the study by Holinger *et al.* [58]. The Bak-BH3 peptide induced apoptosis by binding to the death antagonist Bcl-x_L and preventing its anti-apoptotic effects in HeLa cells. The peptide was introduced into the cells by fusion with the penetratin CPP. As evidence of apoptosis, only cells treated with the peptide contained cleaved PARP (poly ADP-ribose polymerase), a product of activated caspases. However, the event itself did not show any early loss of mitochondrial membrane potential or an early detection of cytochrome *c* release into the cytosol. The authors hypothesized that there could be another death agonist protein that the Bak peptide displaces from the Bcl-x_L heterodimer complex that could mediate the induction of apoptosis. Although Apaf-1 is thought to be a likely candidate for such a protein, there is contradictory evidence for the binding of Apaf-1 with Bcl-x_L [59,60].

When introduced into prostate cancer cells by electroporation, the Bak-BH3 peptide enhanced apoptosis in a caspase-dependent manner. The peptide reduced the interaction between endogenous Bak and Bcl-2, suggesting that the peptide attenuates the anti-apoptotic activity of Bcl-2 [61]. The ability of the Bak-BH3 peptide to bind to Bcl-x_L and promote apoptosis was confirmed in terminally differentiated erythrocytes that expressed only Bcl-x_L and Bak proteins [62]. In this study, the cell-permeable Bak-BH3 peptide fused to the penetratin CPP antagonized the pro-survival function of Bcl-x_L by disrupting the calcium homeostasis of the erythrocyte membrane in a caspase-independent mechanism. Brewis *et al.* used a different approach using vectosomes to deliver the Bak-BH3 peptide to Cos-1 and CT26 cells [63]. Vectosomes are particles of assembled complexes based on the structural protein of herpes simplex virus, VP22 and a short fluorescent oligodeoxynucleotide (ODN). Once inside the cells these vectosomes can be redistributed on activation by light, which is dependent on the fluorophor on the ODN molecule. A BH3-22 fusion vectosome was effectively delivered into Cos-1, skeletal muscle cells and CT26 adenocarcinoma cells, and light activation successfully

triggered the apoptotic response. Furthermore, when CT26 cells bearing BH3-22 vectosomes were injected subcutaneously into Balb/C immunocompetent mice, tumor growth was significantly slowed on application of light at the tumor site. Sonoporation using microbubble-enhanced focused ultrasound was used to introduce the Bak peptide into two different cell lines, HeLa and BJAB (human B-cell lymphoma) [64]. This method of transducing the peptide into cells was found to be cell line-dependent as the BJAB cell line showed more cell death compared with the HeLa cell line.

In a separate study, Dharap and Minko engineered a short peptide derived from the BH3 region of Bak [65]. A luteinizing hormone-releasing hormone (LHRH) decapeptide (QHWSYGLRPG) was conjugated to the amino terminus of the Bak-BH3 peptide for specificity and intracellular delivery. As expected, the apoptosis-inducing activity of the LHRH-BH3 fusion peptide was differentially related to the level of LHRH receptors (LHRHR) expressed by cancer cells. With the highest level of LHRHR, the human ovarian carcinoma cell line A2780 was most sensitive to the fusion peptide. With the lowest level of LHRHR, the prostate cancer PC-3 cell line was the most resistant to the peptide. Similarly, the human ovarian cancer cell line SKOV-3, which is inherently negative for the expression of LHRHR, showed no signs of apoptosis when treated with the LHRH-BH3 peptide. LHRH-BH3 induced apoptosis by a combination of two mechanisms. First, it inhibited the anti-apoptotic proteins Bcl-2 and Bcl-X_L by binding to them. Second, it upregulated the expression of apoptotic protease-activating factor-1 (APAF-1) and induced the release of Smac from the mitochondria. These events led to the cleavage and activation of caspase 9 and, eventually, the activation of effector caspase 3.

A new targeted pro-apoptotic anticancer drug delivery system was designed based on the Bak-BH3 peptide, the anticancer drug camptothecin (CPT) and the LHRH peptide [66]. Polyethylene glycol (PEG) was used as a carrier to enhance solubility and bioavailability of this drug delivery system. The CPT-PEG-BH3 complex and CPT-PEG-LHRH complex both showed superior cytotoxic- and apoptosis-inducing properties compared with free camptothecin or even the camptothecin-PEG complex in ovarian cancer cells, A2780. The proposed drug delivery system has a dual function to reinforce the anticancer effect of camptothecin: i) the LHRH peptide offers specificity and targets the anticancer drug, camptothecin to ovarian cancer cells that have overexpressed LHRH receptors; and ii) the BH3 peptide antagonizes the effect of the anti-apoptotic proteins allowing camptothecin to induce cell death. Furthermore, the antitumor activity of the drug delivery system consisting of CPT-PEG-BH3 was confirmed in an athymic ovarian cancer mouse model, and a correlation between tumor reduction and induction of apoptosis was observed [67]. These results qualify the proposed drug delivery system for further development as a new technology to advance current chemotherapy.

A 19-mer Bak-BH3 peptide, delivered by the penetratin peptide, decreased head and neck squamous cell carcinoma cell proliferation [68]. Following 1 h of treatment with the peptide, cytochrome *c* release was detected in immunoblots. Four hours after treatment with the peptide, induction of apoptosis was confirmed by annexin V-FITC staining. Cleaved PARP was also detected, which supports further the pro-apoptotic activity of the Bak-BH3 peptide.

Molecular dynamics simulations of Bcl-X_L complexed with Bak-BH3 peptide reiterates the importance of the α-helical region of Bak from which the BH3 peptide is derived. This region contains conserved hydrophobic residues that are essential for interactions with the hydrophobic cleft of the Bcl-X_L protein [69].

2.2.2.2 *BH3 peptide from Bax*

A 21-mer peptide from the BH3 region of Bax (**Table 1**, peptide 13) was found to inhibit the dimerization between Bax/Bcl-X_L and Bcl-X_L/Bcl-X_L with an IC_{50} of 5.2 and 4.4 μM, respectively. Inhibition of Bax/Bcl-2 and Bcl-2/Bcl-2 by the peptide was weaker with an IC_{50} of 18.7 and 29.6 μM, respectively [55]. Inhibitory studies done with a set of nested peptides, from residues 52 – 72 to 59 – 72, revealed that down to a length of 18 amino acids (residues 58 – 72) the peptide retained its inhibitory function. However, when the length of the Bax peptide was shortened to contain residues 59 – 72, the peptide lost its ability to inhibit dimerization between Bax/Bcl-X_L and Bcl-X_L/Bcl-X_L. This indicates that there is a minimal length requirement for the binding of Bax-BH3 peptides to these complexes. Furthermore, the native BH3 region is essential for binding of the peptide to the dimers because a single mutation, G67R, eliminates the inhibitory function of the peptide. Biologically, binding of the Bax-BH3 peptide translated to a sharp increase in caspase activation within 1 h in *Xenopus* egg extract [57]. This study was done with a 16-mer Bax-BH3 peptide (amino acids 57 – 72). A pro-apoptotic 20-mer Bax-BH3 peptide (residues 55 – 74) induced a loss of mitochondrial transmembrane potential that was accompanied by cytochrome *c* release in isolated mitochondria [70]. Peptide-mediated cytochrome *c* release was inhibited under calcium-depleted conditions, suggesting that the process involved mitochondrial membrane potential. The pro-apoptotic effect of Bax-BH3 peptides could involve binding and disrupting anti-apoptotic protein complexes in the mitochondria. Mutating C62 to serine in a 15-mer Bax BH3 peptide (residues 59 – 73) resulted in a more stable peptide with pro-apoptotic activity [61]. Treatment of apoptosis-resistant prostate cancer cells with this mutant Bax-BH3 peptide resulted in an enhancement of apoptosis in a caspase-dependent manner. Peptides were introduced into cells by electroporation. The killing activity of the peptide is probably due to attenuation of the suppression of apoptosis by Bcl-2, because in the presence of peptide there is a complete inhibition of interaction between endogenous Bak and Bcl-2.

Polster *et al.* used a peptide comprising amino acids 53 – 86 of the BH3 domain of Bax [71]. This peptide (**Table 1**, peptide 14) induced cytochrome *c* release in stable cells expressing marginal amounts of Bcl-2 protein and in murine cells that did not express any Bcl-2 protein. This effect was seen in a dose-dependent manner. The release of cytochrome *c* was not selective, as adenylate kinase was also found to be released by treatment with the peptide. In stable cells expressing Bcl-2, the peptide failed to induce cytochrome *c* release at low concentrations. However, at the highest concentration of the peptide used, there was a small increase in cytochrome *c* release, indicating that Bcl-2 could be partially inhibiting peptide-mediated cytochrome *c* release. In all these cell systems, endogenous Bcl-X_L was also detected, suggesting that perhaps Bcl-X_L also has a role in preventing cytochrome *c* release. Furthermore, peptide-mediated cytochrome *c* release was found to be dependent on the presence of endogenous Bax in cells. Only cells that have endogenous Bax were susceptible to peptide-mediated cytochrome *c* release. Cytochrome *c* release was followed by the appearance of endogenous Bax in the mitochondrial membrane fraction. One of the hallmarks of cells undergoing apoptosis is the integration of Bax protein into the outer mitochondrial membrane. Although Bax insertion in the mitochondrial membrane did not compromise the permeability transition of the inner mitochondrial membrane, the outer mitochondrial membrane was permeabilized in the presence of the Bax-BH3 peptide.

Further support that the Bax-BH3 peptide promotes apoptosis by releasing pro-apoptotic Bcl-2 proteins from complexes with anti-apoptotic Bcl-2 proteins comes from the study by Moreau *et al.* [72]. In isolated mitochondria, the peptide did not physically target Bax to the mitochondria, as evidenced by its inability to induce conformational change in Bax, which is a requirement for mitochondrial translocation. However, it is clear from this study that the Bax-BH3 peptide disrupts the Bax/Bcl-X_L complex, allowing Bax to translocate to the mitochondria. This is followed by cytochrome *c* efflux. When microinjected into rat fibroblasts, the peptide caused cell death consistent with cytochrome *c* release.

Penetratin-mediated delivery of the Bax-BH3 peptide targeted the peptide to head and neck squamous carcinoma cells [68]. Consistent with this finding, the peptide disrupted the Bax/Bcl-2 complex and inhibited cell proliferation. The peptide-induced caspase activation and the cleavage of the caspase product PARP are evidence of the pro-apoptotic activity of the peptide.

An extensive study done with Bax-BH3 peptides of varying lengths identified a minimum length of 15 amino acids for pro-apoptotic activity [73]. Additionally, the amino terminal amino acids to the BH3 core were found to be more critical than the carboxyl terminal amino acids.

2.2.3 BH3 peptides from BH3-only pro-apoptotic proteins

The BH3-only pro-apoptotic proteins include Bad, Bik, Bim and Bid. They promote apoptosis by antagonizing the anti-apoptotic Bcl-2 proteins. They function upstream of caspase activation and facilitate the pro-apoptotic activities of the multi-domain proteins Bax and Bak (**Figure 2**).

2.2.3.1 BH3 peptide from Bad

A 26-amino-acid peptide from the BH3 region of the mouse Bad protein or a peptide from the homologous region of human Bad (**Table 1**, peptides 15 and 16) were found to be sufficient for binding with the death antagonist Bcl-X_L [74,75]. Binding of the peptide leads to the displacement of Bax and Bak from complexes with Bcl-X_L and prevents further complex formation of Bcl-X_L.

A small molecule decanoic acid, $CH_3(CH_2)_8CONH$, was used as a cell-permeable moiety for the intracellular delivery of a short peptide derived from residues 140 – 165 of the BH3 domain of the BH3-only protein, Bad [76]. The peptide with the cell-permeable moiety was termed cpm-1285. It showed binding with the anti-apoptotic Bcl-2 protein with increased affinity in a cell-free system compared with the peptide alone. This characteristic of the complex was attributed to the extra hydrophobic contacts made by cpm-1285 with Bcl-2. Cellular internalization and localization to the cytoplasm of cpm-1285 peptide was rapid, occurring within 15 min of treatment. Consistent with this finding, cpm-1285 induced apoptosis in HL60 cells after 15 min of treatment. Induction of apoptosis was evident by the presence of active caspase 3 and active fragments of PARP. Although cpm-1285 caused a significant loss in HL60 cell viability, it had very little cytotoxic effect in the normal human peripheral blood lymphocytes (PBL). This selectivity of cpm-1285 for tumor cells was supported further by the finding that it also reduced cell viability of 397 pre-B leukemia cells as well as HL60 cells overexpressing Bcl-X_L. Severe combined immunodeficient mice challenged with HL60 cells and treated with cpm-1285 intraperitoneally gained 11 more days of survival over mice that were left untreated. In addition, cpm-1285 did not demonstrate any *in vivo* toxicity in normal C57BL/6J mice. These *in vivo* studies support that cpm-1285 relieves tumor burden without causing general toxicity. However, there are concerns with the short biological half-life of cpm-1285 that could curtail its full potential to reduce tumor growth. Chemical alteration of the peptide has been suggested to address this issue. Regardless, cpm-1285 induced apoptosis primarily in leukemia cells expressing the anti-apoptosis proteins Bcl-2 and Bcl- X_L, and it had tumor-reducing properties.

A similar Bad-BH3 peptide (**Table 1**, peptide 17) fused to the penetratin peptide significantly inhibited 32D and HeLa colony formation, which was accompanied by a loss of mitochondrial membrane potential and an increase in the production of reactive oxygen species [77]. The Bad-BH3 peptide also stimulated Bax activity. However, this was seen only in the presence of Bcl-X_L, suggesting that the apoptotic role of the BH3 peptide is to promote apoptosis by releasing the multi-domain apoptotic proteins from the inhibitory holds of the anti-apoptotic proteins [56,72]. The pro-apoptotic

activity of polyarginine-mediated delivery of the Bad-BH3 peptide was established in various neuroblastoma cell lines [78]. Induction of apoptosis was caspase-dependent. Furthermore, in conjunction with a peptide derived from the BH3 region of Bid, intratumoral injection of the Bad-BH3 peptide significantly reduced neuroblastoma tumor xenografts in mice.

Penetratin-mediated delivery of the Bad-BH3 peptide decreased head and neck squamous cell carcinoma cell proliferation [68]. Following 1 h of treatment with the peptide, cytochrome *c* release was detected in immunoblots. Four hours after treatment with the peptide, induction of apoptosis was confirmed by annexin V-FITC staining. PARP cleavage was also detected, which further supports the pro-apoptotic activity of the Bad-BH3 peptide.

2.2.3.2 *BH3 peptide from Bim*

A version of the polycationic Tat CPP composed of D-amino acids and a glutamine to ornithine substitution (RKKRRO rnRRR) was used to carry a short peptide derived from the BH3 domain of the BH3-only protein Bim (**Table 1**, peptide 18) [79]. The Tat peptide translocated through the cell membrane and facilitated the cellular internalization of the Tat-Bim construct in mouse T-cell lymphoma (EL4), pancreatic cancer (Panc-02) and melanoma (B16) cell lines. Following this, the Tat-Bim fusion peptide induced apoptosis in these cells by means of activation of caspase 3. In addition, low dose radiation and Tat-Bim had additive effects on apoptosis induction, outlining this combination for use in adjuvant therapy. In tumor reduction studies, Panc-02 or B16 subcutaneous tumor-bearing C57BL/6 mice showed temporal delay in tumor growth following intratumoral treatment with Tat-Bim. In survival studies, tumor-bearing animals in the Tat-Bim treatment group had significant survival benefit. More importantly, Tat-Bim did not show *in vivo* toxicity.

2.2.3.3 *BH3 peptide from Bid*

Early on, it was shown that a 16-mer peptide from the BH3 region of Bid protein (**Table 1**, peptide 19) could cause a rapid increase in caspase activation in *Xenopus* egg extract [57]. Walensky *et al.* describe a technique termed hydrocarbon stapling, which stabilizes a peptide's secondary structure by linking two non-neighboring amino acids with a hydrocarbon chain. Hydrocarbon stapling of the Bid-BH3 peptide (**Table 1**, peptide 20) increased its protease resistance and enhanced its cell permeability, which allowed the peptide to activate apoptosis in leukemia cells and cause cell death [80]. The peptide did so by binding with the death antagonist Bcl-2 proteins. In addition, the hydrocarbon-stapled peptide inhibited the growth of human leukemia xenografts *in vivo*. The pro-apoptotic activity of polyarginine-mediated delivery of the Bid-BH3 peptide was established in various neuroblastoma cell lines [78]. Induction of apoptosis was caspase-dependent. Furthermore, together with a peptide derived from the BH3 region of Bad, intratumoral injection of the Bid-BH3 peptide significantly reduced neuroblastoma tumor xenografts in mice. Elegantly designed studies by Oh *et al.* showed that membrane targeting of the Bid-BH3 peptide is necessary for translocating Bax to the mitochondria and triggering its apoptotic activity [81]. The hydrocarbon-stapled Bid-BH3 peptide that was shown to interact with Bcl-X_L and activate the function of Bax and was also shown to target Bax to the mitochondrial membrane [82].

2.2.4 *Smac peptide from the Smac/Diablo protein*

A Smac peptide (**Table 1**, peptide 21) derived from the N terminus of a mature Smac protein has been shown to be necessary for binding to XIAP (X-linked inhibitor of apoptosis proteins) [83]. Only the first four residues, AVPI (**Table 1**, peptide 22), make protein contact with the BIR3 domain of XIAP [83,84]. Fulda *et al.* showed a synergistic effect of a synthetic Smac peptide on TRAIL-induced apoptosis in malignant glioma cells [85]. Cellular uptake of the Smac peptide was facilitated by conjugation of the peptide with the Tat CPP. By targeting XIAP, the Smac peptide disrupted the inhibition of XIAP on caspase 3 and the apoptosome complex, thereby promoting apoptosis. The peptide synergized the apoptotic stimuli of various death receptors, including TRAIL, CD95 and the chemotherapeutics doxorubicin and cisplatin in a dose-dependent manner. The Smac peptide also sensitized several apoptosis-resistant cell lines, including SHEP/Bcl-2, SH-SY5Y, Mel-HO and Panc-1, to the apoptotic stimulus of TRAIL or doxorubicin. More importantly, in a human glioma xenograft model, the effect of Smac in combination with optimal concentration of TRAIL was tumoricidal. Inhibition of tumor growth was free of any neurotoxicity, and this was seen in the enhanced survival of animals in this treatment group. Interestingly, the effect of the Smac peptide and TRAIL was limited to tumor cells and did not include normal cells, providing a strong case for the use of the Smac peptide and TRAIL for the treatment of apoptosis-resistant cancer cells.

Penetratin has also been used for the intracellular delivery of the Smac peptide [86]. This fusion peptide binds to XIAPs and cIAPs and releases the bound caspase 3 for the activation of apoptosis. The study showed that the Penetratin-Smac polypeptide synergizes the apoptotic effect of diverse chemotherapeutics, including paclitaxel, etoposide, doxorubicin and 7-ethyl-10-hydroxycamptothecin (SN-38). These studies were conducted in a panel of breast cancer cell lines as well as a cholangiocyte cell line.

The apoptotic effect of polyarginine peptide (R8)-delivered Smac peptide (7 amino-terminal residues, **Table 1ss** peptide 21) was investigated in three non-small lung cancer cell lines, including NCI-H460, which has deficient apoptosome activity that correlates with increased levels of XIAP [87]. The R8-Smac peptide at 50 μM was not cytotoxic to any of the non-small lung cancer cell lines used in the study. However, treatment of the H460 cells with R8-Smac peptide sensitized these cells to the apoptotic effect of the antineoplastic agents

cisplatin and paclitaxel. Furthermore, the R8-Smac peptide synergized the antitumor effect of cisplatin in the treatment of H460 tumors in mice. Heckl *et al*. used a FITC-labeled polyarginine peptide to transport the seven amino acids of the NH2 terminus of the Smac peptide across the cell membrane [88]. They conjugated the Smac peptide with the carrier peptide by means of an ss-Alanine linkage. This delivery method altered the subcellular localization of the Smac peptide. FlTC fluorescence was seen mostly in the nucleus. Even so, nuclear uptake was accompanied by cell death. In other examples, 7-mer and 8-mer N-terminal peptides of Smac fused with penetratin have been used to sensitize various cancer cells to the apoptosis-inducing effects of chemotherapeutics, TNF-α, TRAIL or irradiation [89-92].

One should be careful, however, about selecting the CPP and the concentration used to deliver the Smac peptide. At low concentrations, the cationic CPPs penetratin, Tat and polyarginine (R9) provided protection against the pro-apoptotic effect of the cargo Smac peptide in apoptosis-induced HeLa cells [93]. HeLa cells pretreated with the penetratin-Smac peptide at concentrations < 40 μM were resistant to caspase 3 activation on exposure to apoptotic stimuli from TNF and cyclohexamide. At these concentrations of the penetratin-Smac peptide, cell viability was not affected by TNF or cyclohexamide. The cationic CPPs, at least the ones included in this study, compensated for the pro-apoptotic effects of the Smac peptide by mediating the dynamin-dependent internalization of the TNF receptors and preventing subsequent downstream signaling. Therefore, these findings imply that the CPPs can influence activity of the cargo protein and affect cellular signal transduction.

Cytoplasmic delivery of the Smac peptide can be achieved by a cytoplasm-transducing version of the Tat peptide [94]. The nuclear localization property of the Tat peptide is altered dramatically by substitution of several key residues of the α-helical wheel. The triple mutant Tat peptide (K4R, K5A and Q8R) translocates to the cytoplasm in a mechanism different from the wild-type peptide. Although the initial binding of the peptide to the negatively charged cell membrane is crucial for peptide internalization, internalization itself is not associated with cholesterol-based lipid-raft mediate endocytosis. A condition where apoptosis is inhibited was created by transfecting Jurkat cells with a plasmid expressing XIAP followed by UV-irradiation. The Smac peptide that was delivered by the cytoplasm-transducing Tat peptide into these cells resulted in a significant reduction of cell survival compared with the wild-type Tat peptide. As the Smac peptide has a cytoplasmic target, this new Tat peptide is suitable for its cellular delivery.

The Smac/DIABLO protein exists as a dimer. A dimeric Smac N-terminal peptide (**Table 1**, peptide 23) is necessary to relieve the downstream effector caspase 3 from the BIR2 domain of XIAP. The peptide does so in a cooperative manner with respect to the release of caspase 9 from the BIR3 domain of XIAP [95]. The linker region connecting BIR1 to BIR2 of XIAP binds to the active site of the effector caspases 3 and 7 and inhibits them. It turns out that this interaction is followed by the binding of the N terminus of caspase 7 to the Smac-binding region on BIR2. So the binding of the Smac peptide to BIR2 not only limits the exposure of the linker region, but also competes with the effector caspases directly for the IAP binding pocket on BIR2 [96]. Also, it was shown that a bivalent Smac mimetic peptide binds to both BIR2 and BIR3 domains, thus effectively blocking both caspase 9 and caspases 3 and 7 interactions with XIAPs [97]. The bivalent peptide was a more potent antagonist of XIAP compared with its monovalent counterpart, as shown by the enhancement in displacement of caspases 3, 7 and 9 on addition of the bivalent Smac peptide.

3. Conclusion

The work reviewed here demonstrates the therapeutic potential of cell cycle inhibitory and pro-apoptotic peptides. The design of these peptides can be easily manipulated, and they are specific inhibitors of the target proteins for which they are designed. The main caveat for the use of peptides for therapy is their intratumor/intracellular delivery. Although in cell culture studies transfection reagents and electroporation techniques are routinely used to introduce peptides into the cells, these are not feasible *in vivo*. As discussed in this review, cell-penetrating peptides are the most widely used tools to translocate therapeutic peptides into cells. Other approaches include sonoporation and vectosome-based delivery.

4. Expert opinion

This review highlights the major developments in generating peptide inhibitors of cell proliferation, specifically by blocking cell cycle progression or inducing apoptosis. The cell cycle inhibitory peptides described function by inhibiting cyclin/Cdk activity either by mimicking a natural Cdk inhibitory protein or by interfering with cyclin/Cdk binding to target proteins. This is a promising approach because of the frequency of mutation of either p53 (which controls expression of the Cdk inhibitor p21) and/or p16 in many human cancer types. These peptides could be useful in these tumor types where normal cell cycle regulation is impaired. The same is true of the pro-apoptotic peptides reviewed, which function by mimicking pro-apoptotic proteins and inhibiting anti-apoptotic proteins. Part of the transformation process of many cancer cells includes inactivation of these apoptotic cascades, and the use of peptides to reactivate them is a promising approach. As with the signal transduction-inhibiting peptides, successful application of the cell cycle inhibitory or pro-apoptotic peptides depends on the ability to identify the molecular characteristics of patients' tumors and tailor the therapy to each tumor individually. For example, patients with p53 mutation or deletion will probably benefit from therapy with p21-based peptides, and patients with tumors that overexpress

the anti-apoptotic Bcl-2 or Bcl-X_L may benefit from therapy with peptides from the pro-apoptotic Bcl-2 family proteins.

The common theme that arises when discussing all TPs is the need for the development of improved drug delivery techniques to make these molecules viable as drugs. To take advantage of the great potential in TP therapy, ways to prevent peptide degradation and deliver peptides to a therapeutic site, be it a tumor or other diseased site, need to be developed. As discussed in this review, the use of alternative amino acids or chemical modifications such as hydrocarbon stapling, are effective ways to combat the problem of peptide degradation. Furthermore, specific peptide delivery to the tumor site can be improved by the use of macromolecular carriers. The most versatile approach might be to use multifunctional macromolecular carriers that combine elements for active tumor targeting and for extravasation and tumor penetration. Active targeting can be achieved using stimulus-responsive polymers [98,99], antibodies [100], tumor-specific ligands [101], tumor-homing peptides [102,103], or any other means of conferring a macromolecular peptide carrier with a specific affinity for the tumor tissue. Once targeted to the tumor, the carrier must be capable of delivering the TP out of the vasculature and into the tumor cells. There is evidence that CPPs can mediate extravasation of cargo molecules [104,105], and CPPs have even been reported to deliver cargo across the blood–brain barrier [106]. If the problems of lability and poor cell penetration can be overcome using a combination of CPPs, alternative amino acids, macromolecular carriers and active targeting approaches, TPs may emerge as one of the most specific, easily designed and broadly applicable classes of drugs in our anticancer arsenal.

Declaration of interest

D Raucher is President of Thermally Targeted Therapeutics (TTT), a private company working to commercialize ELP drug delivery technology. GL Bidwell III is a paid consultant of TTT.

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Affiliation

Drazen Raucher†, Shama Moktan, Iqbal Massodi & Gene L Bidwell III
†Author for correspondence
The University of Mississippi Medical Center, Department of Biochemistry, 2500 North State Street, Jackson, MS 39216, USA
Tel: +1 00 1 601 984 1510;
Fax: +1 00 1 601 984 1501;
E-mail: draucher@biochem.umsmed.edu